

Original article

Effects of single nucleotide polymorphisms in β -amylase1 alleles from barley on functional properties of the enzymes ☆

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Abstract

Differences in extractable β -amylase (1,4- α -D-glucan maltohydrolase, EC 3.2.1.2) activities exist between enzymes from malt and feed barleys (*Hordeum vulgare* L.). Others have documented few differences in the β -amylase (*bmy1*) gene coding regions and large differences in the non-coding regions of genomic DNA for endosperm specific β -amylases from malt and feed barleys. Researchers correlated differences in intron III with different activity levels in the seeds. Here we demonstrate that differences in the coding regions of *bmy1* from a malt ('Morex') and a feed ('Steptoe') barley significantly affected the biochemical properties of the enzymes. We evaluated the contributions of the three single nucleotide polymorphisms that caused differences in the amino acid sequences at positions 115, 165, and 430. Two polymorphisms (positions 115 and 430) were found to impact the enzymatic activity of β -amylase. The effect of the single nucleotide polymorphism at position 165 was dependent upon the identity of the amino acid in position 115. The contributions of the three single nucleotide polymorphisms to β -amylase thermostability varied with assay temperature. These biochemical results explain the contribution of the favorable *bmy1* allele from the feed barley Steptoe to the quantitative trait loci found on chromosome 4H at the *bmy1* locus.

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1. Introduction

A key criterion in the determination of whether or not barley seeds are of malt quality, a value-added trait for which a market premium is paid, is the seeds' ability to convert starch to fermentable sugars. The seed enzymes responsible for starch conversion include α -amylase, β -amylase, α -glucosidase, and limit dextrinase. The industrial conversion of starch to sugars typically occurs between 63 and 75 °C. These elevated temperatures are necessary to gelatinize the starch, which renders it more susceptible to enzymatic attack [1]. The high temperatures, however, cause

thermal denaturation of several of the amylolytic enzymes, including β -amylase [19], which results in reduced efficiency of starch degradation. One goal of malting and brewing research has been to find or create a more thermostable or more active β -amylase. A more active enzyme, even if thermolabile, would result in more catalysis prior to denaturation than presently occurs.

Barley β -amylases, α -1,4-exohydrolases (EC 3.2.1.2) that release maltose from the non-reducing ends of α -glucans, are encoded by two genes. One β -amylase gene (*bmy2*) on chromosome 2H encodes a ubiquitous β -amylase that is abundant in non-seed tissues and either absent [10] or expressed in very low amounts in seeds [20]. The other β -amylase gene (*bmy1*), located on chromosome 4H, encodes endosperm-specific β -amylase [10]. Three distinct *bmy1* alleles affect the enzymatic activity levels and thermostabilities of β -amylases from malting and feed quality barley cultivars. Eglinton et al. [2] and Kihara et al. [8,9], studying Australian germplasm or very diverse *Hordeum* germplasm, respectively, found three *bmy1* alleles that correlated with enzymes that had low,

Abbreviations: QTL, quantitative trait loci; rBmy1, recombinant β -amylase 1; SNP, single nucleotide polymorphism.

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intermediate, and high levels of thermostability. Erkkilä et al. [3] found few differences in the coding regions and large differences in a non-coding region, specifically the 5' end of intron III, of genomic DNA from three alleles of *bmy1*. They correlated differences in seed β -amylase activities with structural differences in genomic DNA for the *bmy1*s from a diverse group of *Hordeum* germplasm. They attributed this correlation only to differences in intron structure and not to other possibilities, e.g. regulation of gene expression or differences in the primary structure of the enzymes.

There were two objectives of the work presented here. The first objective was to determine if the single nucleotide polymorphisms (SNPs) we document in the coding region of *bmy1* alleles from 'Morex', a malting barley cultivar, and 'Steptoe', a feed barley cultivar, resulted in differences in the biochemical properties of the resultant enzymes. The *bmy1* alleles from these two cultivars were shown by Erkkilä et al. [3] to have differences in intron III structures such that the gene from Morex was categorized as one encoding a high activity enzyme and that from Steptoe to be a gene encoding a low activity enzyme. If the coding regions of these two *bmy1* alleles were shown to result in phenotypic differences between the resulting enzymes, the second objective was to determine which of the SNPs were responsible for these differences. The first objective was addressed by subcloning the *bmy1* cDNAs, isolated from Morex and Steptoe, and expressing them in *Escherichia coli*. The resulting recombinant β -amylases were purified and characterized. The second objective was addressed by using site directed mutagenesis to change the identity of the amino acids encoded by the SNPs from one allele to the identity of the other allele and examining the effect of these changes upon the activity and thermostability of the mutated enzymes.

2. Results and discussion

2.1. Comparison of recombinant wild type β -amylases from Morex and Steptoe

The amino acid sequences deduced from the cDNAs for *bmy1* from the cultivars Morex and Steptoe are shown in Fig. 1. These two sequences differ only by the amino acids in positions 115, 165, and 430. Examination of the cDNA sequences (not shown) revealed a total of five nucleotide differences; however, nucleotide substitutions encoding amino acids in positions 177 and 234 were silent. The amino acid sequences in Fig. 1 were compared to those from the barley cultivars Franklin, Schooner, Adorra, and Haruna nijo (not shown). None of these sequences varied from another by more than five amino acids (0.9% variation). This agrees with Erkkilä et al. [3] who documented very little variation among the amino acid sequences deduced from the coding regions of *bmy1* genomic DNA from a feed barley, a malting barley and *Hordeum vulgare* sp. *spontaneum* PI296897, which has very high β -amylase activity.

Mx	MEVNVKGNVQVYVMLPLDAVSNNRFEKGDELRAQLRKLVEAGVDGVMV	50
St	MEVNVKGNVQVYVMLPLDAVSNNRFEKGDELRAQLRKLVEAGVDGVMV	50
Mx	DVWVGLVEGKGPAYDWSAYKQLFELVQKAGLKLQAIMSFHQCGGNVGD	100
St	DVWVGLVEGKGPAYDWSAYKQLFELVQKAGLKLQAIMSFHQCGGNVGD	100
Mx	VNIPQPQVRDVGTCDPDI FYTDGHGTRNIEYLTGLVDNQPLFHGRSAVQ	150
St	VNIPQPQVRDVGTCDPDI FYTDGHGTRNIEYLTGLVDNQPLFHGRSAVQ	150
Mx	MYADYMTSFRENMKDFLDAGVIVDIEVGLGPAGEMRYPSPQSHGWSFPG	200
St	MYADYMTSFRENMKDFLDAGVIVDIEVGLGPAGEMRYPSPQSHGWSFPG	200
Mx	IGEFICYDKYLQADFKAAAAAVGHPEWEPFNDVGQYNDTPERTQFFRDNG	250
St	IGEFICYDKYLQADFKAAAAAVGHPEWEPFNDVGQYNDTPERTQFFRDNG	250
Mx	TYLSEKGRFFLAWSNNLIKHGDRILDEANKVFLGYKVLAIKISGIHWW	300
St	TYLSEKGRFFLAWSNNLIKHGDRILDEANKVFLGYKVLAIKISGIHWW	300
Mx	YKVPASHAAELTAGYYNLHNRDGYRTIARMLKRHRASINFTCAEMRDSQS	350
St	YKVPASHAAELTAGYYNLHNRDGYRTIARMLKRHRASINFTCAEMRDSQS	350
Mx	SQAMSAPEELVQVLSAGWREGLNVACENALPRYDPTAYNTILRNARPHG	400
St	SQAMSAPEELVQVLSAGWREGLNVACENALPRYDPTAYNTILRNARPHG	400
Mx	INQSGPPEHKLFPGFTYLRSLNQLVEGQNYANFKTFVDRMHANLPRDPYVD	450
St	INQSGPPEHKLFPGFTYLRSLNQLVEGQNYANFKTFVDRMHANLPRDPYVD	450
Mx	PMAPLPRSGPEISIEMLQAAQPKLPFPFQEHDTLPVGPTGGMGGAQEG	500
St	PMAPLPRSGPEISIEMLQAAQPKLPFPFQEHDTLPVGPTGGMGGAQEG	500
Mx	PTCGMGQVKGPTGGMGGAEDPTSGMGGELPATM*	535
St	PTCGMGQVKGPTGGMGGAEDPTSGMGGELPATM*	535

Fig. 1. Aligned deduced amino acid sequences of β -amylase1 from Morex (Mx) and Steptoe (St) barley cultivars. The three amino acid differences between the two sequences are highlighted.

A comparison of the kinetic parameters of the purified recombinant β -amylases (rBmy1), produced from cDNA cloned from Morex and Steptoe seeds and expressed in *E. coli*, is presented in Table 1. The purity of these enzymes is demonstrated in Fig. 2. The few single nucleotide differences present in the cDNAs of the *bmy1* genes from Morex and Steptoe have large effects on the kinetic parameters of the expressed proteins (Table 1). Erkkilä et al. [3], based on the structure of intron III, predicted the β -amylase from Steptoe would have lower activity than the enzyme from Morex. However, we found that the rBmy1 from Steptoe had a k_{cat} that was 67% greater than that of the enzyme from Morex. The K_m s for maltopentaose of these two enzymes were also very different. The K_m for maltopentaose of the rBmy1 from Morex was approximately 40% lower than that of the en-

Table 1
Kinetic parameters for Morex and Steptoe recombinant wild type β -amylases

	Amino acids at SNP positions			K_m (mM)	k_{cat} (s^{-1})
	115	165	430		
Morex rBmy1	C	E	A	0.55	1248
Steptoe rBmy1	R	D	V	0.93	2090

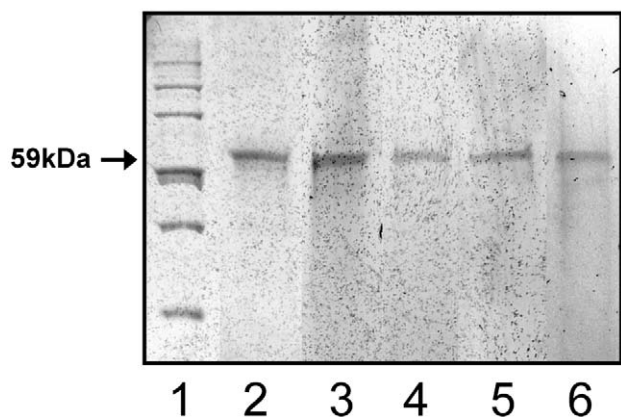


Fig. 2. SDS-polyacrylamide gel showing purity of recombinant β -amylases. Lanes contain: (1) precision unstained broad range protein standards ranging from 25 to 150 kDa (Bio-Rad, Hercules, CA); (2) Steptoe wild type; (3) Morex wild type; (4) R115C mutant; (5) D165E mutant; and (6) R115C/D165E double mutant β -amylases.

zyme from Steptoe and from allelic forms of rBmy1 from the Australian cultivars Franklin and Schooner [12]. Recent work by Ma et al. [13] showed that sequence differences were responsible for differences in the K_m s for reduced potato starch, but had no effect on the k_{cat} s of the rBmy1s from Franklin, a malting barley cultivar and Schooner, which is typically used as feed barley. Of these two kinetic parameters, the more important one in determining which allelic form of β -amylase is preferred for malting barleys is the k_{cat} , as a more rapid catalytic turnover rate will result in the conversion of more substrate to product within a given period of time. Interestingly, a quantitative trait loci (QTL) for diastatic power (a collective measurement of malt carbohydrase activities) has been identified on chromosome 4H in a 3.3 cM interval flanked by the structural gene of *bmy1* in the Steptoe \times Morex double haploid mapping population [4,5]. The favorable allele was shown to be contributed by Steptoe [5]. The contribution of this favorable QTL allele from Steptoe may result from the phenotype of the Bmy1, shown here to be more catalytically active than the enzyme from Morex.

2.2. SNP effects on β -amylase activity

To determine what effect the SNPs had on the activities of the β -amylase enzymes, site directed mutagenesis of the Steptoe cDNA was done to alter the amino acids at positions 115 and 165, singly or jointly, to be the same as those in the β -amylase from Morex. The enzymatic activities, expressed as $\mu\text{kat mg}^{-1}$ purified rBmy1, of the wild types and mutated rBmy1s are shown in Table 2. The R115C (C_{115} , D_{165} , V_{430}) mutant enzyme showed a 38% reduction in activity compared to its parental wild type, Steptoe (R_{115} , D_{165} , V_{430}). Ma et al. [13] showed that having either an R or a C in this position had no effect on the β -amylase activity. However, the rBmy1 sequence they studied, cloned from Schooner, differs from the Morex and Steptoe sequences by having an L in position 347 and an I in position 527, where Morex and Steptoe rBmy1s have an S and an M, respectively. Hence, our

Table 2

Enzymatic activity levels of the two wild types and three mutated recombinant β -amylases. Hydrolysis rates reported are means \pm S.D. of three separate assays

	Amino acids at SNP positions			(μkat mg ⁻¹ Bmy)
	115	165	430	
Morex rBmy1	C	E	A	8.9 \pm 0.04
Steptoe rBmy1	R	D	V	9.4 \pm 0.15
R115C	C	D	V	5.8 \pm 0.06
D165E	R	E	V	10.0 \pm 0.09
R115C/D165E double mutant	C	E	V	10.3 \pm 0.29

results are not directly comparable. The D165E (R_{115} , E_{165} , V_{430}) mutant enzyme showed activity comparable to its parental wild type enzyme, Steptoe (R_{115} , D_{165} , V_{430}) (Table 2). Although no preference for either a D or an E in position 165 was exhibited when the residue in position 115 was an R, when a C was in position 115, the identity of residue 165 was important. The combination of C_{115} and D_{165} (R115C mutant rBmy1) resulted in a 35–45% loss of activity compared to the enzymes with C_{115} and E_{165} (Morex wild type rBmy1 and R115C/D165E double mutant rBmy1).

Assessment of the impact of the SNP at position 430 was done by comparing activities of R115C/D165E (C_{115} , E_{165} , V_{430}) rBmy1 double mutant to the Morex wild type rBmy1 (C_{115} , E_{165} , A_{430}) as these two proteins only differ by the identity of the residue at position 430. The R115C/D165E (C_{115} , E_{165} , V_{430}) rBmy1 double mutant was 16% more active than the Morex (C_{115} , E_{165} , A_{430}) rBmy1. While a V at position 430 was important, it was not able to overcome the deleterious effects of the combination of a C at position 115 and a D at position 165 (R115C mutant rBmy1). Ma et al. [13] found no difference in enzyme activity regardless of whether the amino acid in position 430 was a V or an A. Again, the rBmy1 sequences used in their study differ from those of Morex and Steptoe rBmy1 sequences in positions 347 and 527, and these sequence differences may account for the different results obtained when evaluating the importance of a V or an A in position 430.

2.3. Thermostabilities of recombinant wild type β -amylases

The test criterion selected to compare the thermostability of different enzymes is important. The most commonly used criterion is the T_{50} , which is the temperature at which 50% of an enzyme's activity remains. However, the T_{50} s of barley β -amylases typically do not vary greatly (Table 3) [2,6,12,13]. Kaneko et al. [7] found the percent activity surviving an exposure to 57 °C to be a better criterion for detecting variation in thermostabilities among barley β -amylases than the T_{50} and Kihara et al. [8,9] used 57.5 °C to detect differences in barley β -amylase thermostabilities. Although we observed no significant difference in the percent activities remaining after exposure to 57 °C between the

Table 3

The T_{50} s and the percent residual activities remaining at 57 and 60 °C for the Morex and Steptoe recombinant wild type β -amylases and the three mutated β -amylases

	Amino acids at SNP			T ₅₀	% residual activity	
	positions					
	115	165	430		57 °C	60 °C
Morex rBmy1	C	E	A	59.3	82.3	40.0
Steptoe rBmy1	R	D	V	59.8	85.6	46.7
R115C	C	D	V	58.3	71.3	21.7
D165E	R	E	V	58.3	69.7	21.4
R115C/D165E (double mutant)	C	E	V	59.8	86.0	46.9

^a Significant difference at the $P < 0.01$ level; ^b significant difference at $P < 0.05$ level; ^c no significant difference

wild type rBmy1s from Morex and Steptoe, a significant ($P < 0.05$) difference in survival after exposure to 60 °C was observed. At this temperature, the Steptoe rBmy1 was 7% more thermostable than the Morex rBmy1 (Table 3; Fig. 3). Again, the only differences between these two enzymes are the amino acids at positions 115, 165, and 430; therefore, the thermostability differences must be caused by these amino acid variations. Kaneko et al. [7] identified a QTL for β -amylase thermostability on chromosome 4H in the Steptoe \times Morex double haploid mapping population and attributed the favorable allele to Steptoe. We show here that the Steptoe rBmy1 has a higher thermostability than the rBmy1 from Morex and attribute the thermostability QTL for β -amylase observed by Kaneko et al. [7] to the higher thermostability inherent to the Steptoe Bmy1 protein itself.

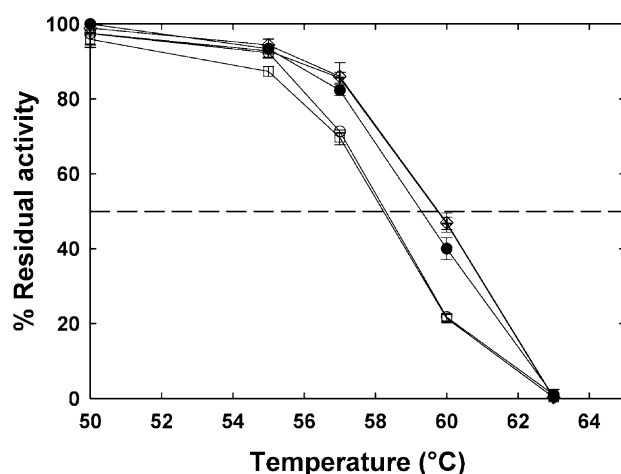


Fig. 3. Profiles of activities as a function of temperature of the Morex and Steptoe wild types and the three mutant recombinant β -amylases. The residual activity remaining at each temperature was calculated as a percentage of the activity remaining at 0 °C which was set to 100%. The dark circle represents the Morex wild type, the cross hair represents the Steptoe wild type, the open circle represents the R115C mutant, the open square represents the D165E mutant, and the open diamond represents the R115C/D165E double mutant enzyme. The error bars represent S.D.

2.4. SNP effects on β -amylase thermostability

The effects of SNPs upon the thermostability of the Morex and Steptoe rBmy1s were examined by changing the amino acids in positions 115 and 165 of Steptoe to the identity of those in Morex. A comparison of the thermostabilities of the R115C (C_{115} , D_{165} , V_{430}) and the D165E (R_{115} , E_{165} , V_{430}) mutant enzymes with the Steptoe (R_{115} , D_{165} , V_{430}) wild type rBmy1 reveals that the mutant enzymes had significantly lower survival rates after exposure to 57 and 60 °C, yet the T_{50} s were not greatly affected (Table 3). Ma et al. [13] report similar results of the R115C and D165E mutations on the T_{50} s of the Schooner β -amylase. In contrast to the results we report, Ma et al. [13] found both the R115C and D165E mutations to be of little consequence to the survival of β -amylase activity after exposure to 57 or 60 °C. The sequence differences in positions 347 and 527 between the different β -amylases studied may account for these observed differences in thermostability.

As was the case for β -amylase activity, the combination of amino acids in positions 115 and 165 was important to enzyme thermostability. There were two deleterious combinations (C_{115} and D_{165} or R_{115} and E_{165}) resulting in decreased thermostability and two positive combinations (R_{115} and D_{165} or C_{115} and E_{165}) resulting in increased thermostability at 57 and 60 °C (Table 3; Fig. 3). Both positions 115 and 165 are on the protein's surface as shown by the crystal structure of the β -amylase from barley [14] and are not near each other. Although the protein surface is considered to be flexible and capable of tolerating changes in the primary structure with minimal loss of activity or thermostability, it is clear that the identities of the residues at positions 115 and 165 in the β -amylase from barley are critical. It is unlikely that the R115C mutation created a stabilizing disulfide bond, as there are no neighboring cysteines to partner with in such a bond. Position 115 is near an α -helix terminus. The R115C mutation removes a positive charge that might have countered the dipole moment at the end of the helix. Although

position 165 is on the surface of the protein and at the end of an α -helix, as is position 115, changing D to E does not alter the charge present as both are negative.

The effect of the amino acid difference at position 430 on the thermostability characteristics of recombinant β -amylase was examined. A comparison of the T_{50} s and the percent activities remaining after exposure to 57 °C for the R115C/D165E (C_{115} , E_{165} , V_{430}) double mutant enzyme and the Morex (C_{115} , E_{165} , A_{430}) wild type rBmy1 shows few differences (Table 3; Fig. 3). However, the percent activity remaining after exposure to 60 °C was significantly increased ($P < 0.05$) for the R115C/D165E double mutant enzyme relative to that of the Morex wild type rBmy1. Having a V at position 430, however, could not overcome the negative effects of $C_{115} + D_{165}$ or $R_{115} + E_{165}$ on the thermostability at 57 and 60 °C.

Ma et al. [12] examined the thermostability profiles of rBmy1s cloned from two Australian barley cultivars, Franklin and Schooner. The Franklin rBmy1 had higher thermostability than the Schooner rBmy1. Ma et al. [13] identified the amino acids at positions 233 and 347 as important for controlling thermostability. The mutants V233A and L347S showed an increase of 1.9 and 2.1 °C, respectively, in T_{50} and combined as a double mutant, there was an increase of 4 °C. Both the Morex and Steptoe rBmy1s sequences have a V at 233 and an S at 347. The T_{50} s of Morex and Steptoe rBmy1s are 2.0–4.6° higher than those reported for the rBmy1s from Franklin and Schooner.

3. Conclusion

SNPs in the coding region affect activity and thermostability levels of the β -amylase enzyme. The rBmy1 from Steptoe has 67% more activity than the rBmy1 from Morex. The only differences between these two enzymes are the three amino acids at positions 115, 165, and 430. The amino acids in the Steptoe sequence responsible for the high level of activity are an R at 115, a D at 165, and a V at 430. With regard to the thermostability of β -amylase, either the combination of C and D or R and E at positions 115 and 165, respectively, results in reduction of activity remaining after exposure to 60 °C. A V in position 430 results in a slight increase in thermostability. These results differ from previous reports that stated that β -amylases from North American cultivars were in the intermediate or low category of thermostability and that the intron III structure was responsible for the β -amylase activity levels. Those studies were primarily done using two-row barley cultivars, which have many differences from six-row cultivars, adapted to different regions of the world and had undergone selections with different end-use criteria.

Studies are currently underway to determine if the higher activity and greater thermostability imparted by the β -amylase allele in Steptoe results in the production of more fermentable sugars during industrial starch conversion. If so, incorporation of the allele from Steptoe into elite malting

barley germplasm should be relatively straightforward through traditional breeding methods.

4. Methods

4.1. Material sources

Morex and Steptoe (*Hordeum vulgare* L.) endosperm-specific *bmy1* cDNA clones were developed by Dr. Fuqiang Chen (Oregon State University) and donated to Dr. Ron Skadsen (USDA-ARS Cereal Crops Research Unit, Madison, WI), who provided them for use in this project. Oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, CA). Chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise specified.

4.2. Sequencing

The cDNAs cloned from Morex (Genbank Accession AF414081) and Steptoe (Genbank Accession AF414082) were sequenced using the Sanger method [16] with an automatic sequencer by the Interdisciplinary Center for Biotechnology Research, University of Florida-Gainesville, FL. All mutations created were confirmed by the same method.

4.3. Site directed mutagenesis

Site directed mutagenesis was done using the Muta-Gene Phagemid In Vitro Mutagenesis Version 2 kit from Bio-Rad Laboratories (Hercules, CA). The oligonucleotide ATGTGTG-GCTGCTGGGTAGGGCGGA removed the stop codon that originally existed at the 5' end of the *bmy1* cDNAs isolated from the Morex and Steptoe cultivars. The oligonucleotide GTCGGGATCACACGTGCCGACGTCCCGCACC changed the amino acid R at position 115 to a C (R115C) in the Steptoe cDNA. The oligonucleotide CACCAGCATCCAAAACTC-TTTCATGTTCTCC changed the amino acid D at position 165 to an E (D165E) in the Steptoe cDNA. To generate the R115C/D165E double mutant, the R115C oligonucleotide was used in combination with the cDNA encoding the D165E mutant.

4.4. Expression in *E. coli*

The *bmy1* cDNAs from Morex and Steptoe seeds were subcloned into the pET21b expression vector (Novagen, Inc., Madison, WI) at the BamHI/EagI/NotI sites in the multiple cloning site. The C-terminal His tag that is encoded by the pET vector was not expressed due to an internal stop codon within the *bmy1* cDNAs. After expression of rBmy1 proteins was induced by 1 mM isopropyl β -D-thiogalactopyranoside, the cultures were grown for 6 h at 37 °C in an orbital shaking incubator. Cells were pelleted by centrifugation (4000 \times g, 20 min, 4 °C) and then resuspended in cold 20 mM Tris-HCl (pH 8). The suspension was centrifuged a second time under the same conditions and the resulting cell pellets were frozen

at -80°C . Cells were lysed with the BugBuster protein extraction reagent (Novagen, Inc., Madison, WI) using a ratio of 5 ml reagent to 1 g of wet cell paste. The slurry was shaken at room temperature for 20 min and cellular debris was removed by centrifugation ($16,700 \times g$, 20 min, 4°C). The supernatant was dialyzed overnight at 4°C against 50 mM sodium acetate (pH 5.2) in preparation for affinity chromatography and thermostability studies.

4.5. Purification of recombinant wild type and mutant β -amylases

α -Cyclodextrin affinity columns were made using the method of Vretblad [17]. One column for each wild type and mutant enzyme was created to avoid any possibility of cross contamination. The affinity chromatography protocol used was similar to that of Yoshigi et al. [18] with the following exceptions. After the second α -cyclodextrin column, the fractions containing β -amylase activity were combined and concentrated on a Centricon Plus-20 (Millipore Corporation, Bedford, MA) according to the manufacturer's instructions. The concentrated supernatant was applied to the same α -cyclodextrin column as used in the first two chromatography steps, this time in the absence of any ammonium sulfate. The β -amylase activity eluted off the column immediately and the fractions containing activity were assessed for purity using SDS-PAGE [11] and silver staining (Bio-Rad Silver Stain Plus kit, Bio-Rad Inc., Hercules, CA).

4.6. Determination of total protein concentration

The total protein concentrations of recombinant purified β -amylases were determined using the BCA Protein Assay kit (Pierce, Rockford, IL).

4.7. Assay of enzymatic activity

Activities of β -amylase were assayed at 40°C using either maltopentaose or the *p*-nitrophenyl maltopentaoside substrate provided in the β -amylase enzyme specific kit Betamyl (Megazyme International Ireland Ltd., Co. Wicklow, Ireland). During protein purification the column fractions were screened for β -amylase activity using the Betamyl kit according to the manufacturer's directions. The Betamyl kit was also used to determine hydrolytic rates of purified recombinant enzymes (Table 2). These assays contained 50 ng purified rBmy1 in 50 mM sodium acetate (pH 5.2) containing 1 mg ml^{-1} BSA. Assays were incubated for 2.5 and either 7.5 or 10 min during which time rates were linear and within the initial velocity range. These assays were done in triplicate.

4.8. Determination of kinetic parameters

Determination of K_m and k_{cat} values was conducted with maltopentaose, buffered in 50 mM sodium acetate (pH 5.2), as the substrate. Nineteen substrate concentrations ranging from 0.14 to 11.5 mM were used. Assays, initiated by addition of 7 ng purified rBmy1 from Morex or 4 ng purified

rBmy1 from Steptoe (both in the presence of 1 mg ml^{-1} BSA), were incubated at 40°C for 2.5 and 7.5 min (during which time rates were linear and within the initial velocity range), then terminated by boiling for 5 min. These assays were also done in triplicate. Reaction mixtures were separated on a CarboPac PA-1 column ($250 \times 4 \text{ mm}$, Dionex, Sunnyvale, CA) using a Shimadzu HPLC (VP series HPLC C system) following the method of Muslin et al. [15]. The elution gradient was from 99.5% A (100 mM sodium hydroxide) and 0.5% B (100 mM sodium hydroxide in 600 mM sodium acetate) to 43% A:57% B over 20 min, followed by a 10 min wash with 99.5% A:0.5% B. Separated carbohydrates were detected and quantified with a pulsed electrochemical detector (ESA coulochem II) using a gold electrode. The starting potential, 0.050 V, was applied for 0.4 s and detection at 0.3 s. The cleaning cycle was from 0.41 to 0.61 s at 0.75 V followed by -0.1 V from 0.62 to 1.02 s. Maltose and maltopentaose were identified by co-chromatography with standards. Lineweaver–Burk plots were used to determine K_m , V_{max} , and k_{cat} values. The k_{cat} ($V_{max}/[E]_t$) values were determined using enzyme concentrations calculated based on the molecular masses of the deduced amino acid sequences.

4.9. Assay of enzymatic thermostability

Three separate cultures were used to produce three crude rBmy1 preparations for determination of enzyme thermostability. Crude extracts were prepared as described in Section 4.4. Crude extracts were diluted 1000-fold with 50 mM sodium acetate (pH 5.2) containing 1 mg ml^{-1} BSA. Enzyme extracts were incubated for 10 min at temperatures ranging from 0 to 63°C . After cooling to room temperature, samples were immediately assayed for residual activities using the Betamyl kit. Assays were initiated by addition of substrate diluted in water and were terminated at either 2.5, 7.5 or 10 min as per manufacturer's instructions. Data at each temperature were expressed as a percentage of the activity remaining after incubation at 0°C , which was set to 100%. Student's *t*-tests were used to determine significance (SAS System for Windows Version 8, SAS Institute Inc., Cary, NC).

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